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# Test of Surfactant-Based Dermal Wound Cleansers on Sulfur Mustard-Exposed Human Keratinocytes in an In Vitro Wound Healing Model

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**14. ABSTRACT**

Sulfur mustard is a chemical warfare agent that causes vesication in human skin. These blisters make the victims more susceptible to infection and delay healing of the skin. The first step in treating wounds is to cleanse the wound to prevent infection. Many dermal wound cleaners are commercially available, but little research has been conducted using these products in conjunction with SM-induced cutaneous injury. This study evaluated the impact of using commercially available wound cleansers on SM-induced wounds, in an in vitro wound healing model consisting of normal human epidermal keratinocytes seeded into six-well plates, exposed to sulfur mustard, and wounded (disruption of the cell monolayer) with a sterile wounding instrument. Cells were stained and images captured to measure percent wound fill. Pluronic F-68 surfactant and the dermal wound cleansers Saf-Clens™ and Shur-Clens® were tested. Neither Shur-Clens® nor 10% and 20% Pluronic F-68 surfactant increased wound fill, but the treatments did not harm the cells in the non-wounded areas. Saf-Clens™ killed the cells after one 2-minute treatment. Shur-Clens® may not promote wound healing, but could be safely used, unlike Saf-Clens™, to cleanse sulfur mustard-induced wounds. Other dermal wound cleansers should be used judiciously until proven nontoxic to keratinocytes.

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(1) Objective: Sulfur mustard is a chemical warfare agent that causes vesication in human skin. These blisters make the victims more susceptible to infection and delay healing of the skin. The first step in treating wounds is to cleanse the wound to prevent infection. Many dermal wound cleaners are commercially available, but little research has been conducted using these products in conjunction with SM-induced cutaneous injury. The purpose of this study was to evaluate the impact of using commercially available wound cleansers on SM-induced wounds.

(2) Methods: This study used an in vitro wound healing model consisting of normal human epidermal keratinocytes seeded into six-well plates, exposed to sulfur mustard, and wounded (disruption of the cell monolayer) with a sterile wounding instrument. Cells were then stained and images captured to measure percent wound fill. Pluronic F-68 surfactant and the dermal wound cleansers Saf-Clens™ and Shur-Clens® were tested in this model.

(3) Results: Neither Shur-Clens® nor 10% and 20% Pluronic F-68 surfactant increased wound fill, but the treatments did not harm the cells in the non-wounded areas. In sharp contrast, Saf-Clens™ killed the cells after one 2-minute treatment.

(4) Conclusions: Shur-Clens® may not promote wound healing, but it could be safely used, unlike Saf-Clens™, to cleanse sulfur mustard-induced wounds. However, other dermal wound cleansers should be used judiciously until proven nontoxic to keratinocytes.

## Introduction

Sulfur mustard (SM) is an oily, liquid, persistent chemical agent that can cause injury to the airway, eye, and skin. Initial clinical symptoms in the skin include erythema followed by vesication. The extent of blistering is dependent on the level of exposure to SM. Sulfur mustard (2,2'-dichlorodiethyl sulfide) reacts with nucleophilic sites such as DNA, proteins, and membrane components, and several mechanisms for damage have been suggested.[1] The basal epidermal cell is a primary target for sulfur mustard.[2,3]

Wound healing is a complex and continuous process.[4] Within hours after dermal injury, keratinocytes migrate into the wound. After several days, migrating keratinocytes proliferate, form a protective monolayer over the wound, and participate in epithelialization, a process that reestablishes contact with the underlying basement membrane.[5]

Current treatments for sulfur mustard injuries include deroofing, debridement, irrigation, topical antibiotics, and sterile dressings.[6] However, these treatments need further refinement to eliminate infections, enhance cosmetic outcomes, and improve wound healing. Appropriate in vitro screening techniques, prior to animal testing, may speed up the development of clinical treatments.

The purpose of this study was to test commercially available dermal wound care products in an in vitro wound healing model of SM-induced cutaneous injury, prior to animal testing. This study tested two dermal wound cleansers, Saf-Clens™ and Shur-Clens®, and their active ingredient, Pluronic F-68 surfactant.

## Materials and Methods

### *Cell Cultures*

Normal human epidermal keratinocytes (NHEK) were obtained from Dr. William Smith's laboratory at the U.S. Army Medical Research Institute of Chemical Defense from commercial sources (Cambrex Bioscience, Walkersville, MD), as previously described.[7] Cells (third passage) were seeded into 6-well plates (Corning Corporation, Corning, NY) and grown to 30-40% confluency in Keratinocyte Growth Medium (KGM, Cambrex Bioscience).

### *Sulfur Mustard Wound Production*

Cells were exposed to 0, 5, or 10 µM sulfur mustard (SM), in KGM, in a chemical surety hood for 1 hour.[8] Cells were transferred to an incubator for an additional 2 hours of SM exposure at 37°C and 5% CO2 and then used in the wound healing model. The 3-hour total exposure time was chosen to make this model as clinically relevant as possible and to meet laboratory safety standards for working with SM. A 3-mm wide wound (disruption of the cell monolayer) was created with a sterile wounding instrument.[9,10,11] Cells were rinsed, and the wounded area was examined microscopically to ensure that cellular debris was removed. Wounds were marked on the bottom of the plate with a blue, ultra fine point permanent marker (Sharpie®, Sanford Corporation, Oak Brook, IL), and the wells received fresh media (with or without a surfactant treatment).

*"Rinse" Surfactant Treatments*

For "rinse" surfactant studies, Pluronic F-68, also known as polyoxamer 188 (P1300, Sigma Chemical Company, St. Louis, MO), was diluted in dH2O and filter sterilized (0.2 µm). The commercial wound cleansers Saf-Clens™ (#159712, Convatec, Skillman, NJ) and Shur-Clens® (#121222, Convatec), which contain Pluronic F-68, were used at full strength. NHEKs received daily treatments of vehicle (sterile water), 10% Pluronic F-68, 20% Pluronic F-68, Saf-Clens™, or Shur-Clens® for 2 minutes at room temperature. The treatments were removed and fresh cell culture media, warmed to 37°C, was applied. The culture medium was changed daily for additional control NHEKs that received no treatments.

*"Constant" Surfactant Treatments*

For "constant" surfactant studies, Pluronic F-68 (P1300, Sigma Chemical Company) was diluted in KGM and filter sterilized (0.2 µm) to prepare working stocks. The surfactant was diluted to its final concentrations (0.0125, 0.025, or 0.05 % [wt/vol]) in KGM. Half volume media changes (with fresh surfactant) were performed daily. Control NHEKs received daily half volume medium changes with KGM alone. Half volume media changes were conducted to provide fresh surfactant to cells and to reduce disruption of the cellular environment within the well in the event that cells secreted autocrine factors.

*Crystal Violet Stain*

Cells were stained with the cytoplasmic stain crystal violet (#C3886, Sigma-Aldrich, St. Louis, MO), as previously described.[11] Briefly, cells were fixed with 4% paraformaldehyde, rinsed three times with 0.1M phosphate buffer, and stained for 1 minute in 0.1% crystal violet (wt/vol) in dH2O. Cells were washed with dH2O and air dried.

*Wound Fill Measurements*

Images of stained cells were captured using a digital camera (Coolpix, Nikon Instrument Inc., Lewisville, TX) and a dissecting microscope (#SZX12, Olympus, Center Valley, PA). Images were then analyzed with Image-Pro® Plus 5.0 software (Media Cybernetics, Bethesda, MD) to determine percent wound fill. Each image was analyzed three times.

*Statistical Analyses*

Data are reported as mean ± standard error of the mean (SEM). Statistical significance was defined as $P \leq 0.05$ for all tests. Analyses were conducted using Statistical Analysis System software (SAS Institute, Inc., Cary, NC). Depending on the number of variables involved in a particular study (i.e., SM concentration, drug treatment, day cells stained), a two-sample t-test or an ANOVA was conducted. A Tukey's test was then used to compare the pairs of treatment groups. See figure legends for the specific statistical test conducted.

## Results

*Test of Commercial Dermal Wound Cleansers*

Experiments were performed to evaluate the dermal wound cleansers Saf-Clens™ and Shur-Clens®, and one of the cleaners' ingredients, Pluronic F-68 surfactant (Figs. 1-3). Cells were exposed to either 0 or 10 µM SM, wounds created, and cells treated with 10% Pluronic F-68, 20% Pluronic F-68, Saf-Clens™, or Shur-Clens®. Additional control cells received the F-68 vehicle (H2O) or no daily 2-minute treatments (None). Wells stained immediately after wounding revealed no significant differences between the starting wounds for each treatment group (data not shown).

In the first experiment, cells were stained on day 6 after wounding (Fig. 1). For the 0 µM SM concentration, the 10% and 20% F-68 groups had significantly greater* wound fill than the Saf-Clens™ and vehicle groups (93.62 and 92.19% vs. 10.96 and 0.13%, respectively). The vehicle group had a significantly greater^ wound fill than the Saf-Clens™ group. Similar results were found for the 10 µM SM concentration. The 10% and 20% F-68 groups had significantly greater* wound fill than the Saf-Clens™ and vehicle groups (67.78 and 76.07% vs. 3.52 and 1.4%, respectively). The 20% F-68 group had significantly different^ wound fill than the 10% F-68 group. Microscopic observations revealed that Saf-Clens™ killed the cells after just one 2-minute treatment.

In the second experiment, cells were stained on days 3, 6, and 9 after wounding (Fig. 2). For the 0 and 10 µM SM concentrations, the vehicle control had significantly less*^ wound fill than the Shur-Clens®, 10% and 20% F-68 groups at all days tested. Significant improvements in wound healing were also found on day 9 for the 10 µM SM-exposed cells. Shur-Clens® had significantly greater* wound fill than the 20% F-68, 10% F-68, and vehicle groups (61.59, 34.5, 15.11, and 7.43%, respectively). The 20% F-68 group also had significantly greater* wound fill than the 10% F-68 and vehicle groups.

In the third experiment, NHEK cells were stained on days 3, 6, and 9. For the 0 µM SM concentration, the vehicle and Saf-Clens™ group had significantly less* wound fill than the 10% F-68, 20% F-68, Shur-Clens®, and no treatment groups (0.97 and 2.15% vs. 31.54, 80.17, 75.43, and 81.74%, respectively). The 10% F-68 group also had significantly less* wound fill than the 20% F-68, Shur-Clens®, and no treatment groups. For the 10 µM SM concentration, there was no significant difference in wound fill between any of the F-68 containing products on day 3. On day 6 for the 10 µM SM concentration, the vehicle and Saf-Clens™ had significantly less wound fill than the Shur-Clens®, 20% F-68, and no treatment groups (0.26 and 0.07% vs. 23.52, 29.89, and 38.17%, respectively). Also, the 10% F-68 group had significantly less* wound fill than the Shur-Clens® and no treatment groups (13.36% vs. 29.89 and 38.17%, respectively), and the 20% F-68 group had a significantly less* wound fill than the no treatment group. On day 9 for the 10 µM SM concentration, the vehicle and Saf-Clens™ groups had significantly less$^{+}$ wound fill than the 20% F-68 and no treatment groups (1.01 and 0.93% vs. 18.29 and 32.19%, respectively). Also, the 10% F-68 and Shur-Clens® groups had significantly less$^{+}$ wound fill than the no treatment groups (7.13 and 11.55% vs. 32.19%, respectively).

*Test of Continuous Surfactant Presence*

Additional studies were conducted to see if the addition of Pluronic F-68 to the medium (i.e., "constant" presence) could improve wound healing. In two separate studies, NHEK cells were

exposed to 0 or 5 µM SM (Fig. 4) and 0 or 10 µM SM (Fig. 5), wounded, and treated daily with KGM containing 0.0125, 0.025, or 0.05% F-68. Additional control cells received KGM alone (0% F-68). No significant differences in wound fill were found between any of the F-68 treatment groups on day 8 (Fig. 4) or days 3, 5, 9, and 12 (Fig. 5).

**Figure 1.** NHEK cells exposed to 0 or 10 µM SM and treated (2-min rinse) daily with 10% Pluronic F-68, 20% Pluronic F-68, or Saf-Clens™. Additional control cells received the F-68 vehicle (H2O). Cells were stained on day 6 after wounding. For the 0 µM SM concentration, the 10% and 20% F-68 groups had significantly different* wound fill than the Saf-Clens™ and vehicle groups. The vehicle group had a significantly different^ percent wound fill than the Saf-Clens™ group. For the 10 µM SM concentration, the 10% and 20% F-68 groups had significantly different* wound fill than the Saf-Clens™ and vehicle groups. The 20% F-68 group had a significantly different^ percent wound fill than the 10% F-68 group. Data points represent mean values ± SEM of 3 determinations. A two factor ANOVA was used to compare the SM concentrations and four treatment groups. If a significant interaction of SM concentration and treatment was observed, then a one factor ANOVA at each SM concentration was used to compare the treatment groups. Next, a Tukey's test was used to compare all pairs of treatment groups for significant differences. Statistical significance was defined as $P \leq 0.05$ for all tests.

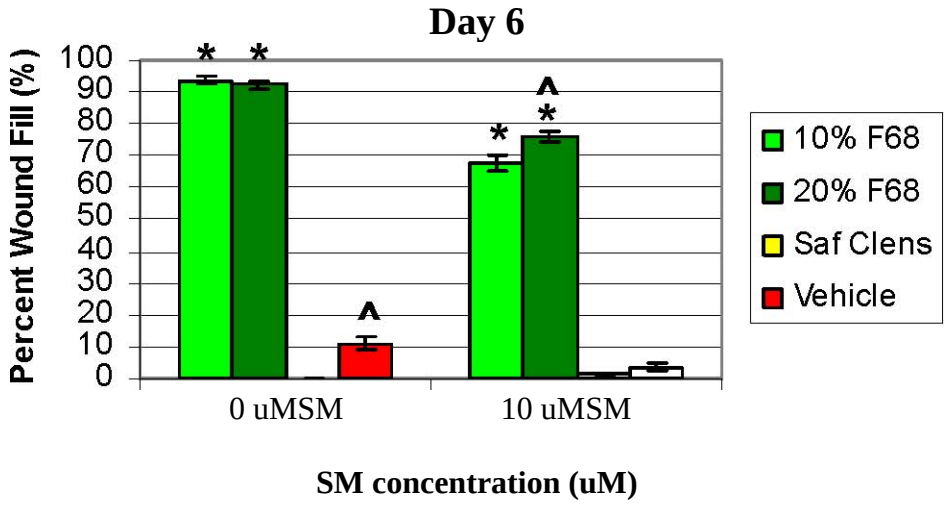
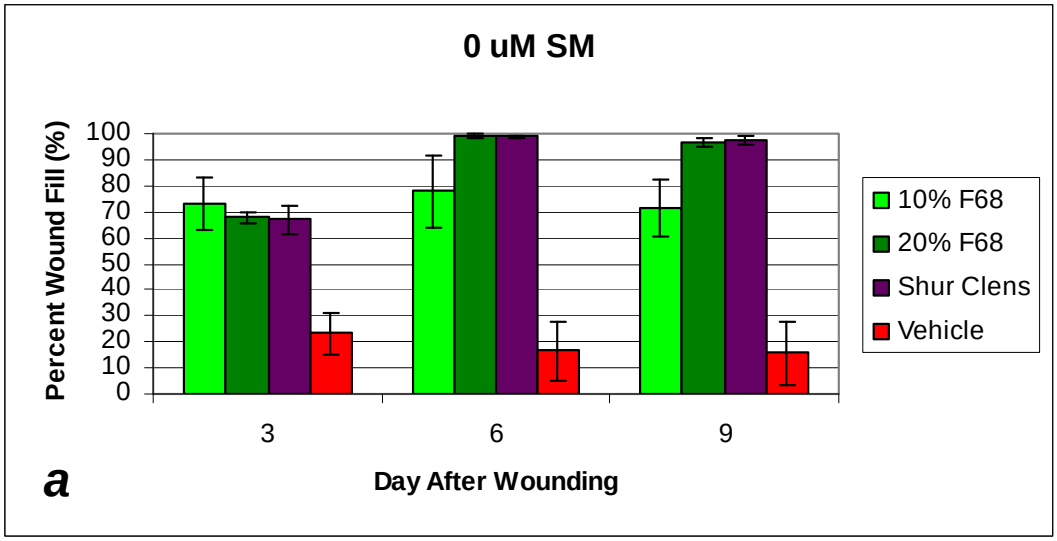


**Figure 2.** NHEK cells exposed to 0 or 10 µM SM and treated (2-min rinse) daily with 10% Pluronic F-68, 20% Pluronic F-68, or Shur-Clens® and stained on days 3, 6, and 9 after wounding. Additional control cells received the F-68 vehicle ($H_2O$). Significant interaction between SM concentration and treatment groups was observed at days 3 and 9. (a) For 0 µM SM, on days 3 and 9, a significant difference* in wound healing was observed between the vehicle and the 10% F-68, 20% F-68, and Shur-Clens® treatment groups. (b) For 10 µM SM on day 3, a significant difference* in wound healing was observed between the vehicle and the 10% F-68, 20% F-68, and Shur-Clens® treatment groups. For 10 µM SM on day 9, significant differences* in wound healing were observed between the Shur-Clens® and the 10% F-68, 20% F-68, and vehicle treatment groups, and between the 20% F-68 and the 10% F-68 and vehicle groups. For both 0 and 10 µM SM, no significant interaction between SM concentration and treatment groups was observed for day 6. However, there were significant differences^ in wound healing between the vehicle and the 10% F-68, 20% F-68, and Shur-Clens® treatment groups and between the 0 and 10 µM SM concentrations. Data points represent mean values ± SEM of 3 determinations. A two factor ANOVA was used to compare the SM concentrations and four treatment groups. If a significant interaction of SM concentration and treatment was observed, then a one factor ANOVA at each SM concentration was used to compare the treatment groups. Next, a Tukey's test was used to compare all pairs of treatment groups for significant differences. Statistical significance was defined as $P \leq 0.05$ for all tests.


0 uM SM
Percent Wound Fill (%)
100
90
80
70
60
50
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3
6
9
Day After Wounding
10% F68
20% F68
Shur Clens
Vehicle
a

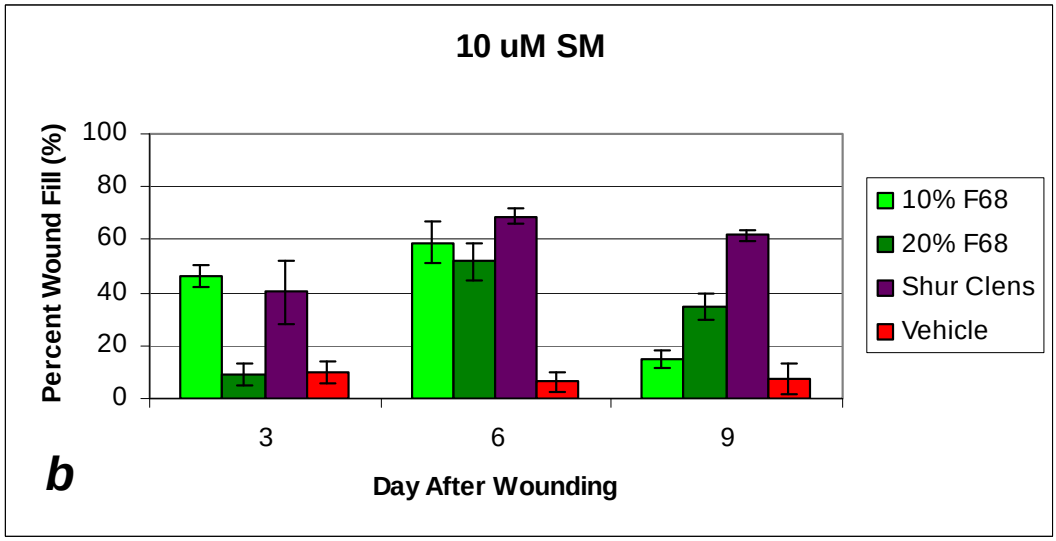
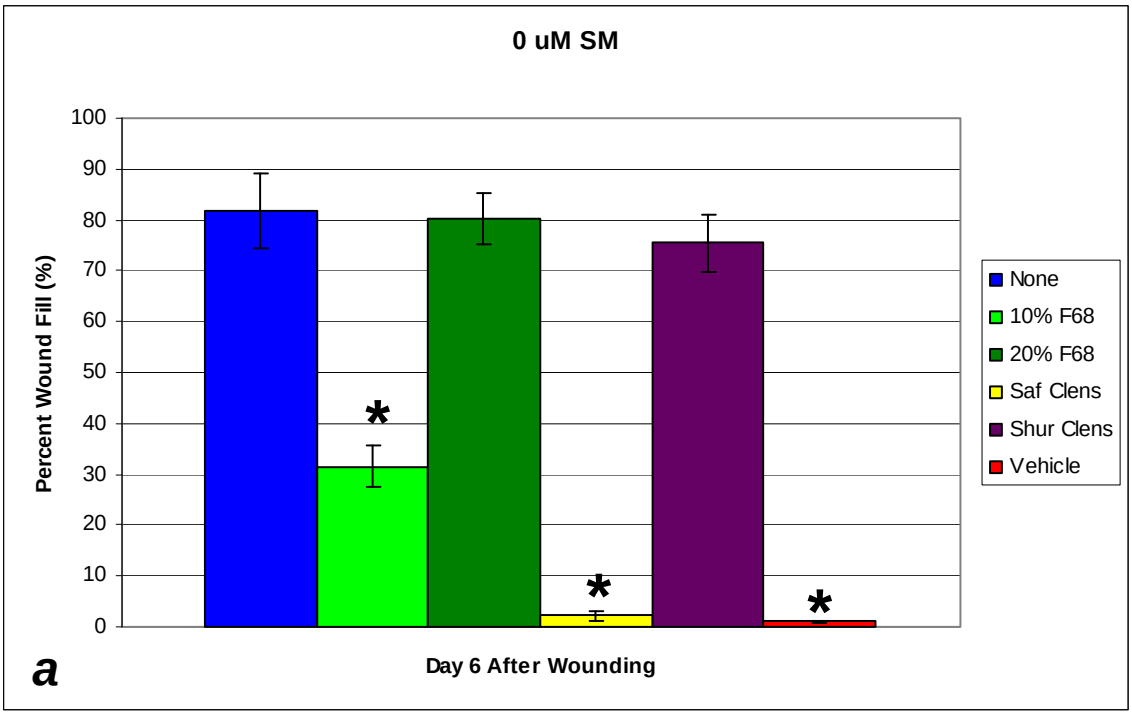

10 uM SM
Percent Wound Fill (%)
100
80
60
40
20
0
3
6
9
Day After Wounding
10% F68
20% F68
Shur Clens
Vehicle
b

**Figure 3.** NHEK cells exposed to SM and treated (2-min rinse) daily with 10% Pluronic F-68, 20% Pluronic F-68, Saf-Clens™, or Shur-Clens®. Additional control cells received the F-68 vehicle (H2O) or no daily 2-minute treatments (None). (a) NHEK cells were exposed to 0 µM SM and stained on day 6. The vehicle and Saf-Clens™ groups had significantly different* wound fill than the 10% F-68, Shur-Clens®, 20% F-68 and no treatment groups. Also, the 10% F-68 group had a significantly different* wound fill than the Shur-Clens®, 20% F-68 and no treatment groups. (b) NHEK cells were exposed to 10 µM SM and stained on days 3, 6, and 9 after wounding. On day 3, a significant difference^ in wound fill was observed between the vehicle and no treatment group. On day 6 for 10 µM SM, the vehicle and Saf-Clens™ groups had significantly different* wound fill than the Shur-Clens®, 20% F-68 and no treatment groups. Also, the 10% F-68 group had significantly different* wound fill than the Shur-Clens® and no treatment groups, and the 20% F-68 group had a significantly different* wound fill than the no treatment group. On day 9, the vehicle and Saf-Clens™ groups had significantly different$^{+}$ wound fills than the 20% F-68 and no treatment groups. Also, the 10% F-68 and Shur-Clens® groups had significantly different$^{+}$ wound fills than the no treatment groups. Data points represent mean values ± SEM of 3 determinations. A one factor ANOVA was used for each staining day/SM concentration combination to compare the treatment groups. If the treatment effect in the ANOVA was significant, then a Tukey's test was used to compare all pairs of treatment groups for significant differences. Statistical significance was defined as $P \leq 0.05$ for all tests.


0 uM SM
Percent Wound Fill (%)
100
90
80
70
60
50
40
30
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10
0
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*
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None
10% F68
20% F68
Saf Clens
Shur Clens
Vehicle
Day 6 After Wounding
a

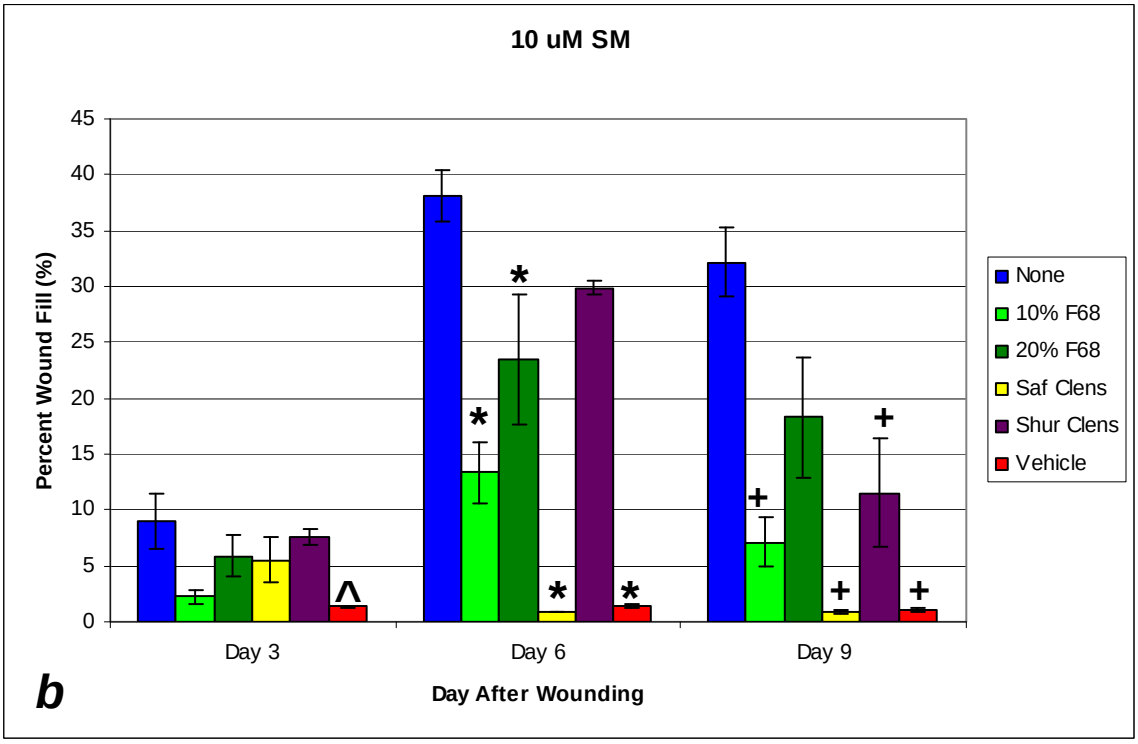

10 uM SM
Percent Wound Fill (%)
45
40
35
30
25
20
15
10
5
0
*
*
*
*
+
+
+
+
Λ
None
10% F68
20% F68
Saf Clens
Shur Clens
Vehicle
Day 3
Day 6
Day 9
Day After Wounding
b

**Figure 4.** NHEK cells exposed to 5 µM SM and replenished daily (constant presence) with 0, 0.0125, 0.025, or 0.05% F-68 in KGM. Cells were stained on day 8 after wounding. No significant differences were observed between F-68 doses and the 0% F-68 (no treatment) group on day 8. Data points represent mean values ± SEM of 3 determinations. A one factor ANOVA followed by a Tukey's test was used to compare all pairs of treatment groups for significant differences. Statistical significance was defined as $P < 0.05$ for all tests.

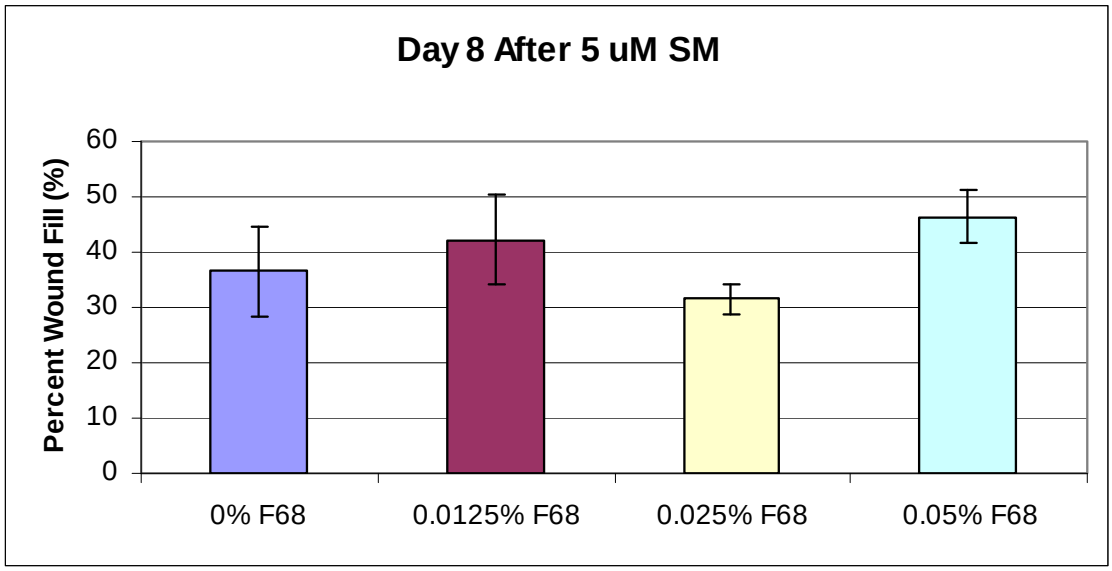
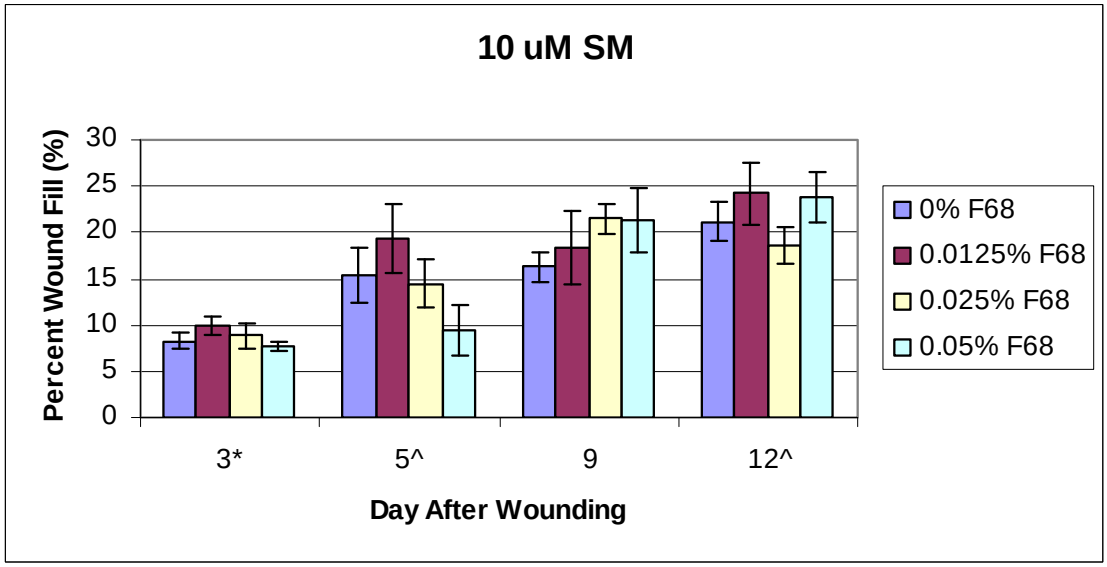



**Figure 5.** NHEK cells exposed to 10 µM SM and replenished daily (constant presence) with 0, 0.0125, 0.025, or 0.05% F-68 in KGM. Cells were stained on days 3, 5, 9, and 12 after wounding. A significant day effect was observed between day 3* and days 5, 9, and 12, and between day 5^ and day 12. No significant differences were observed for the F68 doses. Data points represent mean values ± SEM of 3 determinations. A two factor ANOVA was used to compare the wound healing of F-68 doses and stained days, followed by a Tukey's post hoc comparison of pairs of F-68 doses and pairs of days. Statistical significance was defined as $P < 0.05$ for all tests.



## Discussion

The first step in treating wounds is to cleanse the wound to prevent infection. Migrating keratinocytes also assist in the fight against infection by forming a protective monolayer over the wound.[5] Many dermal wound cleaners are commercially available, but little research has been conducted using these products in conjunction with SM-induced cutaneous injury. The purpose of this study was to use an in vitro wound healing model to evaluate the impact of using commercially available wound cleansers on SM-induced wounds. Normal human epidermal keratinocytes were chosen as they appear to be a good model for basal epidermal cells,[12] a principal target for SM-induced skin lesions.[2,13] The SM concentrations selected for these experiments were based on a previously published study.[11]

Pluronic F-68 surfactant showed promise as an effective wound cleanser in an animal model. Mechanical cleansing of wounds with sponges soaked in Pluronic F-68 prevented wound infections and minimized tissue damage in rabbits.[14] In the present study, two commercially sold dermal wound cleansers, Shur-Clens® and Saf-Clens™, were evaluated in the NHEK wound healing model. Shur-Clens® is a 20% Pluronic F-68 solution made in purified water. Saf-Clens™ contains these main ingredients (although the exact concentration of F-68 is unknown due to proprietary information restrictions) as well as Sorbitol Solution, USP; coco phosphatidyl PG-dimonium chloride; sodium chloride, USP; potassium phosphate monobasic, NF; DMDM Hydantoin; citric acid, USP; and sodium hydroxide, NF added for pH adjustment. Ten and 20% Pluronic F-68 solutions in water were also tested in the model.

Two-minute treatments were conducted to mimic how a patient might use the cleansers (i.e., spray the cleanser on, wait a few minutes for the solution to dry, and then apply a dressing). The Shur-Clens® and F-68 treatments did not improve wound healing, but they also did not harm the NHEK cells in the non-wounded areas. In contrast, Saf-Clens™ damaged and/or killed the NHEK cells just after one 2-minute treatment. The sterile water vehicle control also killed the cells. These findings are consistent with previous studies that showed that Shur-Clens® was nontoxic.[15,16] Wilson et al. used a human infant keratinocyte culture to show that Shur-Clens® was nontoxic and that Saf-Clens™ had to be diluted 10-fold before it reached nontoxic levels in an MTS cell viability assay.[17] Since the Pluronic F-68 solutions did not harm the NHEKs, one can conclude that the toxicity differences between Shur-Clens® and Saf-Clens™ can be attributed to one or more of the extra ingredients contained in Saf-Clens™. One candidate for toxicity is the preservative DMDM Hydantoin (1,3-dimethylol-5,5dimethylhydantoin). In one clinical study, 33% of formaldehyde-allergic patients developed dermatitis after application of a cream containing 1% DMDM Hydantoin.[18] Each ingredient and/or a combination of ingredients must be further tested to definitely state the cause of keratinocyte toxicity. In conclusion, one can concur with Wilson et al. when they suggest that “judicious use of these supposedly innocuous agents (i.e. dermal wound cleansers) should be considered in a clinical setting.”[17]

Previous cell culture studies suggested that the constant presence of surfactant might promote wound healing. Very low dosages of Pluronic F-68 in cell culture media stimulated an increase in the attachment of human gingival fibroblasts (hGF) on plastic and dentin surfaces, suggesting that F-68 may promote early wound healing by facilitating the attachment, spread, and growth of hGFs.[19] The addition of Pluronic F-68 to cell culture media protected periodontal cells from the harmful effects of alcohol. Human gingival fibroblasts were fed with DMEM media containing 0, 1, 2, or 4% ethanol with or without 0, 0.025, 0.05, or 0.1% Pluronic F-68. Cells treated with 0.025 and 0.05% F-68 showed protection of the non-wounded areas of hGFs, with less cell loss

and morphological changes than the ethanol-exposed but untreated hGFs. However, there were no statistically significant improvements in wound fill between the study groups.[20] Similar results were obtained in a study of periodontal ligament fibroblasts fed with DMEM containing 10-fold lower doses of ethanol with or without the Pluronic F-68 concentrations above.[21] In the present study, NHEK cells were exposed to SM, wounded, and replenished daily (constant presence) with 0, 0.0125, 0.025, or 0.05% F-68 in KGM. No significant differences in wound fill were found between any of the F-68 and control treatment groups.

In conclusion, surfactant studies found no improvement in wound fill by Pluronic F-68 surfactant or the dermal wound cleansers Saf-Clens™ and Shur-Clens®. Pluronic F68 and Shur-Clens® did not impair wound healing, but Saf-Clens™ killed the NHEK cells after just one 2-minute treatment. Caution must be used in accepting the advertised safety of products marketed for delicate wound tissue. This in vitro wound healing model will be a useful tool for evaluating additional commercially available wound healing products to combat cutaneous sulfur mustard injuries.

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